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14. **ABSTRACT**

Breast tumor invasion is believed to be triggered by elevated proteolytic enzymes in tumor cells that cause degradation of the basement membrane, whereas results from recent worldwide clinical trials with corresponding enzyme inhibitors have been disappointing. Our studies showed that a subset of pre-invasive breast tumors contained focally disrupted myoepithelial (ME) cell layers, and tumor cells overlying these focal ME layer disruptions showed several unique features, including a significantly higher rate of proliferation, genetic instability, and expression of tumor invasion related genes, compared to adjacent cells within the same duct. Based on these and other findings, we have suggested that focal ME layer disruptions might represent an early sign of tumor invasion, and that cells overlying focal ME layer disruptions might represent precursor of invasive lesions. We have further proposed that breast tumor invasion is triggered by a localized degeneration of injured or aged ME cells, and resultant immunoreactions, which stimulate tumor stem cells to proliferate and invade.

15. SUBJECT TERMS
Breast tumor invasion; Breast tumor progression; Myoepithelial cells; Early detection; Precursor of invasive breast cancer; Estrogen receptor expression; inflammatory cell infiltration.

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Introduction

To assess interactions between epithelial (EP) and myoepithelial (ME) cells in association with breast tumor progression and invasion, a double immunostaining technique with antibodies to smooth muscle actin (SMA) and estrogen receptor (ER) was used to elucidate the ME and EP cells in the breast tissues harboring ductal carcinoma *in situ* (DCIS). Single or clusters of EP cells with a marked diminution or a total loss of the ER expression were found immediately overlying focally disrupted ME cell layers, in sharp contrast to the adjacent dominant population of ER (+) cells within the same duct that showed no associated ME cell layer disruptions. This study attempted to confirm our previous findings on a larger number of cases, and to compare the immunohistochemical and molecular biological profiles of the ER (-) cells overlying disrupted ME cell layers with those of adjacent ER (+) cells and surrounding stromal (ST) cells. Since ME cell layers are physical barriers protecting the microenvironment and integrity of EP cells, and the disruption of ME cell layers is an absolute pre-requisite for breast tumor invasion, the outcomes of this project could have significant values in early detection of breast tumor invasion and/or progression.

Body

a: Statement of work

Task 1. To repeat our previous studies and to identify epithelial (EP) cells overlying disrupted myoepithelial (ME) cell layers (months 1-6)

Using a double immunohistochemical staining method with antibodies to ER and smooth muscle actin (SMA) to simultaneously elucidate tumor and ME cells, our studies examined the structural integrity of ME cell layers in 220 female patients with ductal carcinoma *in situ* (DCIS). Of 5,698 ducts and acini examined, 405 (7.1%) ducts and acini were found to contain focal disruptions (defined as the absence of ME cells resulting in a gap equal to or greater than the combined size of at least 3 ME cells) in their surrounding ME cell layers. The frequencies of ME cell layer disruptions varied significantly among cases (5; Table 1).

Table 1. Frequencies of focal ME cell layer disruptions among different cases

Total cases	No disruptions	About 10% disruption	Over 30% disruptions
220	126 (57.3%)	61 (27.7%)	33 (15.0%)

The frequency of focal basal cell layer disruptions was independent of the size, length, and architecture of ducts or acini, and was also independent of the histological types and grades of tumors (5). About 10% of the disruptions were seen in normal and hyperplastic appearing ducts (5,10,18,19,22,26,30,33).

Focal ME cell layer disruptions appeared to substantially impact the ER expression status in overlying tumor cells. A vast majority of the tumor cells overlying focal ME cell layer disruptions showed a total loss or substantial reduction of ER expression, in sharp contrast to adjacent cells within the same duct but distant from focal disruptions, which were strongly positive for ER. These ER negative cells were generally distributed as clusters and a vast majority of the focal disruptions were overlaid by ER negative cell clusters (5,10,18,19,26,30,33) (Table 2).

Table 2. Correlation between focal ME cell layer disruption and ER expression

Total disruptions	With ER negative cell clusters	With ER positive cell clusters	P
405	350 (86.4%)	55 (13.6%)	< 0.01

In the H&E stained sections, these focal ME cell layer disruptions and the overlying ER negative cell clusters were generally indistinguishable from adjacent morphologically comparable counterparts, and from adjacent cells within the same duct. Under high magnification, however, these ER negative cells differed substantially from their counterparts in nuclear shape, cell density, and polarity (5; Fig 1).

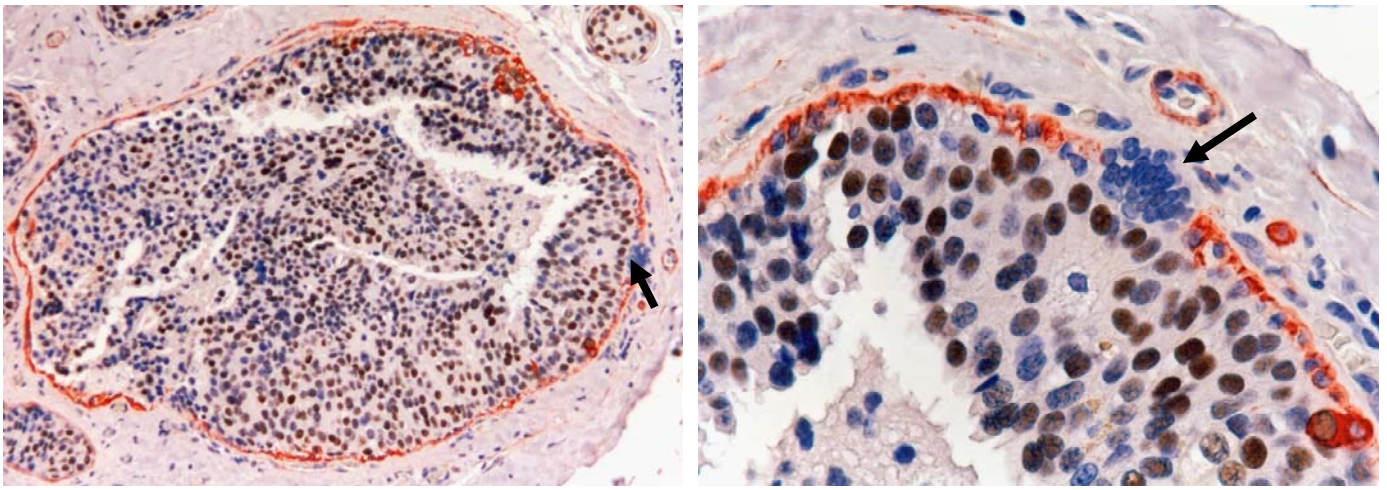

Fig 1. Correlation between focal ME cell layer disruptions and ER expression. Paraffin-embedded human breast tissues (with DCIS) double immunostained for SMA (red) and ER (brown). Note that the cell cluster (arrows) overlying focal ME cell layer disruptions completely lacked ER expression, whereas adjacent cells within the same duct were ER positive.

This task was completed in time, and the outcomes had been published (see attached "References-publications: 5, 10, 18, 19, 26,30,33,57-60).

Task 2. To compare the biological behavior of cells overlying a disrupted ME cell layer with that of adjacent cells within the same duct (months 6-9)

Using a double immunohistochemical staining method with antibodies to SMA and a cell proliferation marker (Ki-67), the cell proliferation index in ducts with and without focally disrupted ME cell layers, and in ER negative cell clusters and adjacent cells within the same duct was statistically compared. In three separate experiments, ducts with focally disrupted ME cell layers and ER negative cell clusters consistently showed a significantly higher cell proliferation index than their counterparts, an average of 19% versus 4%, respectively (12, 25,26). A vast majority of the proliferating cells were located at focal ME cell layer disruptions (12). It is interesting to note, however, that about 10% of the ER negative cell clusters completely lacked expression of all cell proliferation-related markers, in sharp contrast to adjacent cells within the same duct, which showed an elevated cell proliferation (18-19).
This task was completed in time.

Task 3. To microdissect phenotypically different EP cells and the surrounding ME and stromal (ST) cells for molecular biological analyses (months 9-12)

This technical procedure was completed in time, and microdissected tissue samples were subjected to molecular biological assessment, as described bellow.

Task 4. To compare the frequency and pattern of loss of heterozygosity (LOH), and clonality among EP, ME, and ST cells (months 12-20)

Microdissected cells were subjected to DNA extraction and genetic analysis with a total of 15 DNA markers from 5 different chromosomes. Results showed that the ER-negative cell clusters overlying focally disrupted ME cell layers and adjacent ER-positive cells within the same duct but distant from disruptions had a markedly different frequency and pattern of loss of heterozygosity and microsatellite instability at 10 of the 15 markers. These markers included those at chromosomes 3p, 11p, and 13q, which harbor several important tumor suppressors (5,10,24,29,57) (Fig 1).

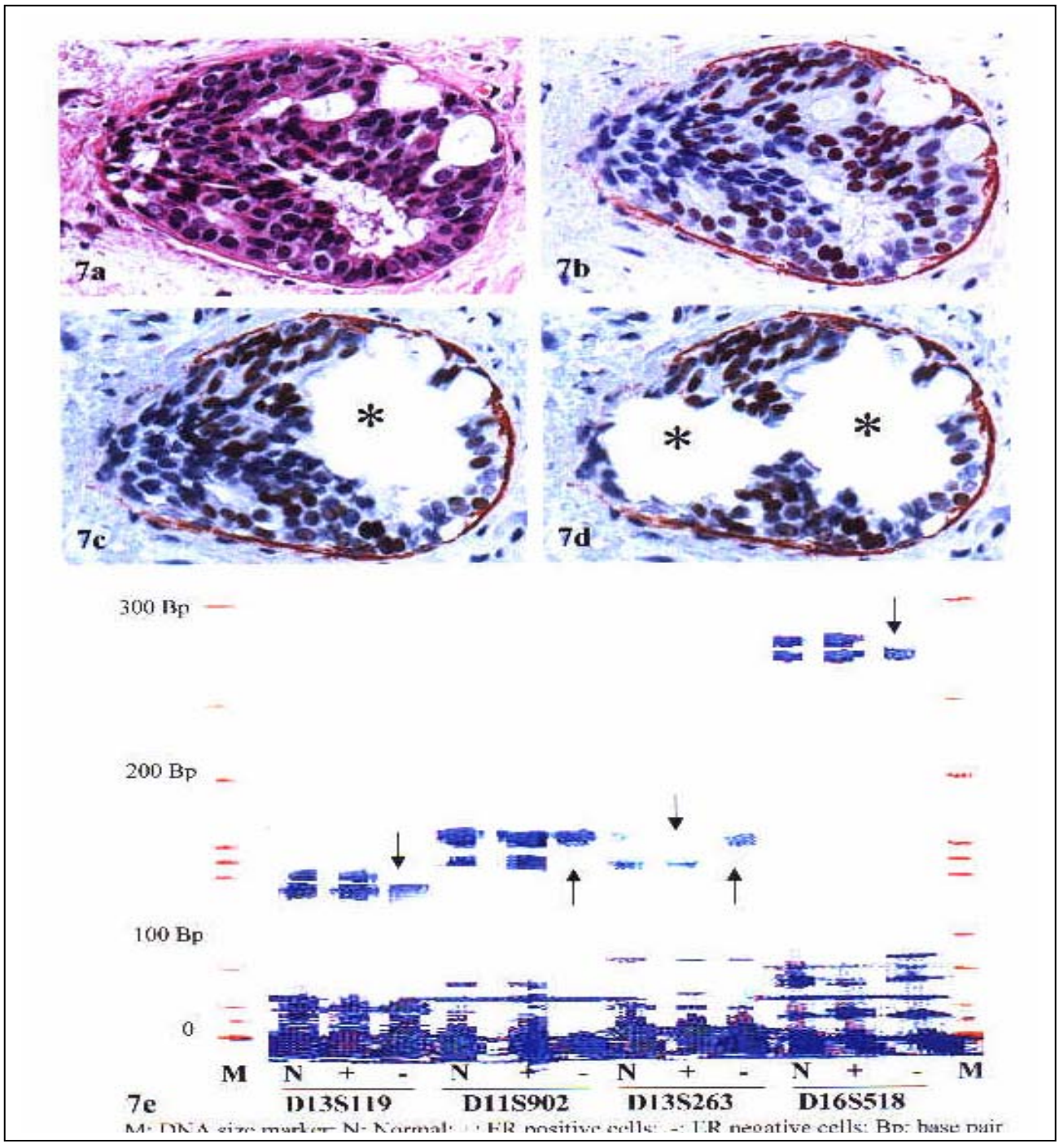

Fig 2. A substantially higher frequency of LOH in ER-negative cell clusters overlying focal ME cell layer disruptions. Microdissected cells were subject to LOH assessment with 15 DNA markers. Asterisks identify cells dissected and arrows identify LOH. Adopted from our published paper: Man et al. Breast Cancer Research 5: R231-241, 2003.

Clonality analysis revealed that a majority of the ER-negative and their adjacent ER-positive cells shared the same mono-clonality, suggestive of malignant nature (Man et al. Unpublished data).

This task was completed in time, and the outcomes had been published (see attached "References-publications: # 6-10 (papers), and #16-27 (abstracts).

Task 5. To assess the gene expression pattern in cells from frozen section sections with cDNA expression array technique, and to generate probes based on sequences exclusively or mainly expressed in cells overlying disrupted ME cell layers (months 20-24)

ER-negative and adjacent ER-positive cells within the same duct were also microdissected in frozen breast tissues from 20 patients with DCIS. Microdissected cells were subjected to gene expression profiling with the cDNA array technology. Results showed that ER-negative cells overlying focally disrupted ME cell layers had a significantly higher expression of cell cycle, and tumor invasion-related genes (10,14,37,57-60) (Fig 3; Table 3).

Table 3. Comparison of the frequency and level of mRNA expression in ER (+) and (-) cells

Gene group	Gene name	Higher in ER (-)	Higher in ER (+)	Equal in both	Case number
Adhesion	CD44	13	4	1	18
	CDH1	13	4	3	20
	MUC18L	5	5	2	12
Angiogenesis	EGFR	5	6	1	12
	IFNA1	10	5	2	17
	TNF	5	9	6	20
Apoptosis	BAX	9	4	0	13
	CASP9	9	5	5	19
	CFLAR	10	5	1	16
Cell cycle	CDC25A	9	1	7	17
	CDKN2A	10	4	2	16
	RAD53	9	1	2	12
Inv & metastasis	NME4	10	4	6	20
	TIMP1	6	6	3	15
Signal transduct	NFKB1	11	6	3	20
Total		134 (54.3%)	69 (27.9%)	44 (17.8%)	247
P			< 0.01		

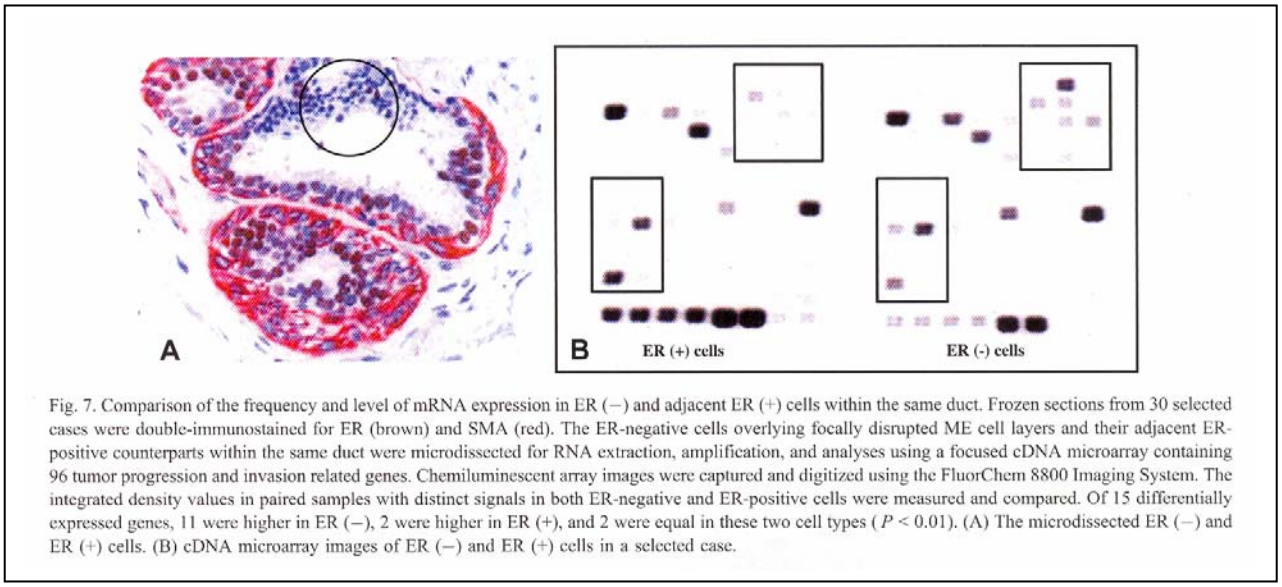

Fig. 7. Comparison of the frequency and level of mRNA expression in ER (−) and adjacent ER (+) cells within the same duct. Frozen sections from 30 selected cases were double-immunostained for ER (brown) and SMA (red). The ER-negative cells overlying focally disrupted ME cell layers and their adjacent ER-positive counterparts within the same duct were microdissected for RNA extraction, amplification, and analyses using a focused cDNA microarray containing 96 tumor progression and invasion related genes. Chemiluminescent array images were captured and digitized using the FluorChem 8800 Imaging System. The integrated density values in paired samples with distinct signals in both ER-negative and ER-positive cells were measured and compared. Of 15 differentially expressed genes, 11 were higher in ER (−), 2 were higher in ER (+), and 2 were equal in these two cell types ($P < 0.01$). (A) The microdissected ER (−) and ER (+) cells. (B) cDNA microarray images of ER (−) and ER (+) cells in a selected case.

Fig 3. A significantly higher expression of cell cycle control and tumor invasion-related genes in ER-negative cell clusters overlying focal ME cell layer disruptions. Adopted from our published paper: Man et al. Breast Cancer Res Treat 89: 198-208, 2005.

Our studies also have also detected several molecules, including beta protein 1 (BP1), atypical E-cadherin and c-erbB2, and cytokeratin-associated proteins, that are exclusively or preferentially associated with breast tumor invasion and progression (15,18,36,42,43,53,55,60,74,85).

Our subsequent studies in patients with co-existing DCIS and invasive breast cancer have further revealed that some of the ER-negative cell clusters showed direct physical continuity with invasive lesions (Fig 4).

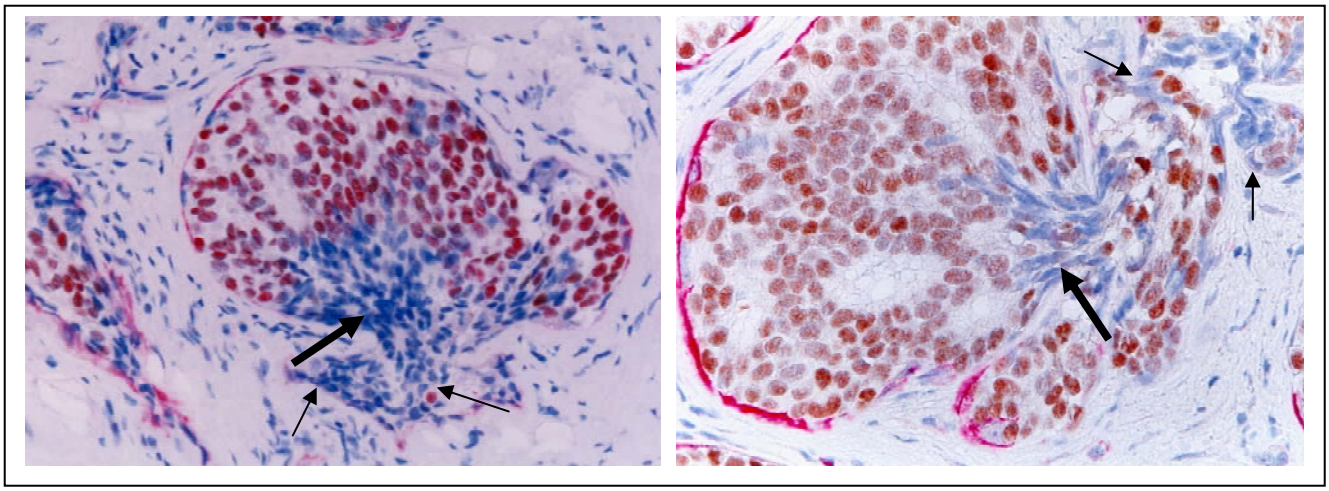

Fig 4. Direct physical continuity of ER-negative cell clusters with invasive lesions. Paraffin-embedded human breast tissue sections double immunostained for SMA (red) and ER (brown). Thick arrows identify ER-negative cell clusters overlying focal ME cell layer disruptions. Thin arrows identify invasive breast lesions.

Based on the above and other findings, we have hypothesized that focal ME cell layer disruptions may represent the early sign on breast tumor invasion, and that ER-negative cell clusters overlying focal

ME cell layer disruptions may represent the direct precursor of invasive lesions. Our hypothesis and supporting data have been published in multiple peer-reviewed journals (5,10,14).

Our recent studies further revealed that ducts and acini with focally disrupted ME cell layers showed a significantly higher rate of leukocyte infiltration than their morphologically comparable counterparts with intact ME cell layers, and a vast majority of infiltrated leukocytes were located near the site of focal ME cell layer disruptions (10,12,52,64,65,67). Focally disrupted ME cell layers showed a significantly high rate of degeneration and apoptosis, and a significantly lower expression of tumor suppressor p63 and PCNA (a proliferation marker) (4,10,104). Our studies in human prostate, cervical, and colorectal tumors revealed similar focal disruptions and related changes (13,20,71-73).

Based on these and other findings, we have further hypothesized that breast tumor invasion is triggered by a localized degeneration of aged or injured ME cells and the resultant auto-immunoreactions. Our hypothesis and supporting data have been recently published in two peer-reviewed journals (10,13,20).

This task was completed in time.

Task 6. To apply the probes to both paraffin and frozen sections, to identify the gene expressing cells and their morphologic features (months 24-32)

Completed: The laboratory procedures have been completed and the outcomes are in the process of summarization for publication (87,88).

Task 7. To correlate the laboratory findings with that of clinical following-up data (months 32-36).

Completed: The laboratory procedures have been completed and the outcomes are in the process of summarization for publication (86).

In addition, the experimental procedures have been recently replicated on more samples using more selected markers, to further validate the results of our completed studies, and to identify more unique molecules expressed in ER (-) cell clusters overlying focally disrupted ME cell layers.

b: Experimental procedures:

Consecutive sections were made from formalin-fixed, paraffin-embedded breast tissues from over 400 patients with various grades of ductal carcinoma in situ (DCIS), and double imunostained for ER and SMA. Cross sections of all ducts lined by ≥ 40 EP cells were examined for a focal ME cell layer disruption, defined as an absence of ME cells, resulting in a gap equal to or greater than the combined size of 3 EP or ME cells. A focal loss of ER expression was defined as marked diminution or a total loss of ER staining in cells immediately overlying a disrupted ME cell layer, in contrast to strong ER expression in adjacent cells within the same duct.

After immunostaining for ER and SMA, cells overlying disrupted ME cell layers, adjacent ER (+) cells within the same duct, adjacent stromal (ST) cells, and other controls were microdissected for DNA extraction and assessment for loss of heterozygosity (LOH) and microsatelite instability (MI), using

PCR amplification with a panel of DNA markers at 6 chromosomes. The frequency and pattern of LOH and MI among samples were compared.

Consecutive sections were also prepared from frozen breast tissues of patients with DCIS and invasive ductal carcinomas (IDC), and were double immunostained for ER and SMA. Immunostained sections were examined for ER expression and focal ME cell layer disruptions. ER (-) cells overlying disrupted ME layers and adjacent (+) cells within the same duct in DCIS, along with morphologically and immunohistochemically similar cells in IDC, were microdissected for RNA extraction, using the RNA extraction kits from Arcturus Engineering, Inc (Mountain View, CA). The RNA extracts were subjected to RT PCR amplification. The gene expression profiles among samples were compared, using the software and reagents from Affymetrix, Inc (Santa Clara, CA) and SuperArray Bioscience Corporation (Frederick, MD).

A total of 7 biotin-labeled probes and detection kits from our collaborators, DAKO Corporation (Carpinteria, CA), and Sigma (St. Louis, MO) had been used in both paraffin-embedded and frozen sections from selected cases. The experimental procedures had been completed and several manuscripts are in preparation to report the results (see "References"-Scientific papers near completion of in preparation).

The clinical follow-up data from 50 cases with focally disrupted ME cell layers had been compared to those from 50 cases without ME cell layer disruptions, and several manuscripts are in preparation to report the results (see "References"-Scientific papers near completion of in preparation).

All above experimental procedures were carried out according to the methods described in the proposal without any major modifications. Also, all the laboratory efforts have been strictly adhered to address the issues listed in "Statement of Work".

Key research accomplishments

All the laboratory procedures for Tasks 1 to 7 had been completed, and the outcomes have been either published or in the process of preparation for publication (see below).

The outcomes of this project have generated 74 published or accepted research papers (n=21), abstracts (n=50), and figures (n=3), as well as 24 submitted (n=6) and partially completed and publishable (n=18) research papers.

Based on his own and other findings, this PI has proposed a new hypothesis for breast and prostate tumor invasion. The hypothesis and supportive data have been recently published in several peer-reviewed journals, including Breast Cancer Research, Breast Cancer Research and Treatment, Experimental Cell Research, Cancer Detection and Prevention, and Applied Molecular Morphology & Immunohistochemistry (see attached "References": Scientific papers published, accepted, and submitted #5, 10, 12-14).

Several molecules exclusively or mainly expressed in ER negative cell clusters overlying focally disrupted ME cell layers have been identified and characterized, and is in the process for potential development of early detection or therapeutic agents.

Reportable outcomes

A total of 103 research papers (n=46), abstracts (n=54), and figures (n=3) are expected to be generated by this projects (see the "References" below).

Conclusions

1. Tasks 1-7 listed in the proposal have been completed.
2. A total of 103 publications are expected to be generated by this project.
3. The outcomes are in a total agreement with the original hypotheses in the proposal.
4. Several new molecules associated with tumor progression or invasion have been identified.

References

A. Honors and Awards:

1. A invited speaker at the Department of Chemistry and Biochemistry at Florida State University in June, 2003
2. Author of one of the best poster presentations at the 7th International Symposium on Predictive Oncology & Intervention Strategies. Nice, France, February 7-10, 2004.
3. Author of one of the best oral presentations at the 7th International Symposium on Predictive Oncology & Intervention Strategies. Nice, France, February 7-10, 2004.
4. A distinguished lecturer at Department of Defense, Center for Prostate Disease Research, October 6, 2004.
5. A invited speaker at the 3rd Annual Drug Discover & Development-Asian-Pacific Congress, June 1-3, 2005, Singapore.
6. A invited speaker at the Drexel University Medical School on September 14, 2005.
7. Invited reviewer for Cancer Therapy in 2004 (one manuscript).
8. Invited reviewer for Cancer Detection and Prevention in 2004 and 2005 (three manuscripts).
9. The PI's work and picture were posted in the AFIP Letter 162 (6): 3, 2004.

B. Research grants:

1. PI of AFIP/ARP joint research initiative grant (05AA) in 2005
2. PI of "Hypothesis Development Award" (PC051308) from Congressionally Directed Medical Research Program in 2005
3. Co-PI of a grant (BCTR05004465) from Susan Komen Breast Cancer Foundation in 2005

C. Patent:

Co-invent of a filed patent (with Dr. Patricia E. Berg of the George Washington University)

D. Publications:

a. Published and accepted scientific papers:

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